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(SYNOPSIS FOR REGISTRATION FOR DEGREE OF DOCTOR OF PHILOSPHY IN BIOTECHNOLOGY)

Proposed Topic	:	Development of SCAR markers for sex determination in *Valeriana wallichii* DC.
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1. Background and Justification:

1a) Sexual system in plants and its implications:

Sexual system refers to the pattern of distribution and function of male and female gamete producing structures (Sakai and Weller, 1999). In animals the predominant type of sexual system is gonochory in which male and female sex functions are restricted to different individuals, which may also be distinct morphologically – a phenomenon known as sexual dimorphism.

In plants, sexual system is more diverse than in animals and manifestations of sexual phenotypes are also varied. Sexual system in plants ranges from hermaphroditism to unisexuality depending upon the occurrence of male and female sex organs in same or different flowers respectively. Furthermore based upon the distribution of unisexual flowers on same or separate individuals, sexual systems like dioecy, monoecy and gynodioecy have evolved in angiosperms. Hermaphroditism is the predominant mode of sexual system found in angiosperms as dioecy or separation of sexes has been reported only in 6% of the known species (Ming *et al.,* 2011). Each type of sexual system has its peculiar implications in success of sexual reproduction and adaptive fitness of the species population. Hermaphroditism which is regarded as the primitive condition, offers the provision of gene flow though both male and female sex tracks i.e., through pollen as well as ovules. It also makes possible the provision of cross as well as self pollination. On the other hand dioecy offers the advantage of better fitness and adaptability of such plants through increased seed set and offspring quality. Another advantage of dioecy is the

prevention of inbreeding depression that may occur frequently in hermaphrodites and affect the seed set and offspring quality.

1b) Sex- Determining mechanisms in plants: Mechanism of sex determination in plant species showing different types of sexual system has been worked out by many workers (Geber, 1999; Matsunaga and Kawano, 2001; Charlesworth, 2002; Barret, 2002).These studies have shown that sex determination may be governed at chromosomal or gene level or may be due to the influence of environmental factors (Westergaard, 1958). These determinants range from sex chromosomes in *Marchantia polymorpha* and *Silene latifolia* to hormonal control in *Zea mays* and *Cucumis sativa.* Role of pheromones in the sex determination of *Ceratopteris richardii* have also been postulated (Tanurdzic and Banks, 2004). It is known that sex is the genetic phenomenon which assures the continuous generation of new gene combinations as substrates for evolution and genetic improvement through breeding. Genetic sex determination may be due to a single locus or multiple tightly linked loci on autosomes or several genes located on heteromorphic chromosomes (Parrish *et al*., 2004; Ming et *al*., 2007). The most extreme type of sex determination system is found in *Cannabis sativa*, *Rumex nivalis, Silene latifolia*, *Carica papaya* and *Asparagus officinalis,* where highly specialized sex chromosomes are found to have an active XY system of sex determination with heterogametic males (XY) and homogametic females (XX). Whether same type of mechanism operates in control of sex or gender in individual flowers in monoecious or dioecious plants is doubtful (Ainsworth *et al*., 1998; Ming *et al*., 2011).

1c) SCAR approach as a tool for sex determination in plants:

The SCAR (Sequence Characterized Amplified Region) based technique was developed by Paran and Michelmore in 1993 for identification of genes imparting resistance against downy mildew in lettuce. It refers to the development of locus specific marker for a genetically controlled trait. Gender/sex specific sequences have been identified using this approach in *Carica papaya* L. (Urasaki *et al.,* 2002; Deputy *et al.,* 2002; Bedoya and Nunez 2007; Niroshini *et al.,* 2008) *Gingko biloba* (Ling *et al*., 2003), *Salix viminalis* L. (Gunter *et al*., 2003), *Ficus fulva* (Parrish *et al*., 2004), *Rumex nivalis* (Stehlik and Blattner, 2004), *Brassica napus* L. (Ke *et al*., 2004), *Humulus lupulus* L. (Danilova and Karlov, 2006), *Broussonetia papyrifera* (Lianjun *et al*., 2012) and *Piper betle* L. (Samantaray *et al.,* 2012). In these studies workers have converted RAPD, ISSR and AFLP amplicons to SCARs. The conversion of RAPD, ISSR and AFLP to SCARs based on sequence data significantly improves the reproducibility and reliability of PCR assays, and therefore, their utility for many applications, such as marker assisted selection and cultivar identification (Busconi *et al.,* 2006).

1d) Plant species proposed for study: *Valeriana wallichii* DC. (Valerianaceae).

Valeriana wallichii DC. (Valerianaceae) is an important medicinal herb distributed in Himalaya from Kashmir to Bhutan between 1200- 3100m (Hooker, 1882; Gilani *et al.,* 2005; Rather *et al.,* 2012; personal observation). Known by the common name 'Indian valerian', this species constitutes a major part of drug trade of India (Anonymous, The Wealth of India, 2008). Rhizome extract of *V. wallichii* is a rich source of Valepotriates which possess several medicinal properties and is used as an active constituent of many ayurvedic and allopathic medicines (Gilani *et al.,* 2005; Mathur and Ahuja, 1991). Plants

are collected from wild on a very large scale. This has led to its overexploitation and the species has been recorded as endangered (Koul and Handa, 2000). Studies on reproductive biology and population structure were carried out in our lab at Centre for Biodiversity Studies, B G S B University which have highlighted some interesting aspects related to sex expression in this species. All the populations studied so far are polygamous consisting of individuals showing 3 distinct patterns of sex expression:

1. Plants bearing only hermaphrodite flowers i.e. hermaphrodite plants.
2. Plants bearing only pistillate flowers i.e. female plants.
3. Plants bearing both hermaphrodite and pistillate flowers in the same inflorescence i.e. Gynomonoecious plants.

This feature of variable sex expression in *V. wallichii* has direct implication on the maintenance of genetic variability and, hence, survival of the species by having a combination of exclusive cross as well as mixed breeding strategies. In addition to this, different sex types of the species differ in the quantity of the active metabolite (Valepotriates) for which the plant is sought for (Unpublished information).

In case of *V. wallichii* however, no studies pertaining to the mechanism of sex determination have been done so far. Hence, the present study is proposed so that the basis for the differential sex expression in this important medicinal plant can become known and sex specific genomic markers be developed which can be helpful in screening of natural populations for commercial as well as conservation purposes.

2. Objectives: The purposed work aims to:

1. Explore the distribution range of the species in parts of the Himalayas to locate as many populations of *V. wallichii* as possible so as to determine the polymorphism in sexual morphs.
2. Develop SCAR markers linked to sex determining trait using a suitable marker system (RAPD/ISSR/AFLP)
3. Revalidate the SCAR markers thus developed by wide screening of known sex types and progenies.

3. Methodology

3a) Collection of plant material: Up to twenty five plants of each sex type depending upon the availability will be collected from all available wild populations and transplanted in the lead botanic garden of BGSB University.

3b.) Sampling will be done during the flowering season spreading from December to May.

3b) Genomic DNA –extraction: Total genomic DNA will be isolated from the fresh leaf samples from all three sex types by following CTAB method proposed by Doyle and Doyle (1990) with modifications whenever required.

3c) Purification and quantification of isolated DNA: The isolated crude DNA may contain several impurities which may interfere during amplification. Thus purification of the crude DNA using 1 or 2 phenolations will be performed if required. Purified DNA will be quantified using visual and spectrophotometric method.

3d) Bulked segregant analysis (BSA). BSA technique (Michelmore *et al*., 1991) will be followed to facilitate identification of molecular markers linked to sex determination in

the species. This technique will be followed with the objective of minimizing or eliminating variation not associated with the specific trait of interest i.e. sex type in *Valeriana wallichii*.

Bulk samples of each sex types will be made separately by pooling an equal amount of DNA isolated from each sex type. Twenty populations from all over the Himalayan region in India will be investigated.

3e) Screening of three sex types using different molecular markers: After purification and quantification, the extracted DNA bulks shall be subjected to marker analysis. The marker systems proposed to be used include: ISSR, RAPD and AFLP.

3f) Conversion of RAPD, ISSR or AFLP markers by their cloning and sequencing into SCARs and to test the specificity of SCAR primers.

It will be achieved by following the below mentioned steps:

a) Excision, cloning and sequencing of a marker

1. Excision of specific amplified fragment/fragments from agarose gel.
2. Purification and enrichment of the excised fragment/s using DNA purification kits.
3. Ligation: the purified and enriched DNA fragment/s will be ligated to some suitable vector depending upon the size of the fragment (pGEM –T Easy vector, pCR 8/GW/TOPO Vector, pTZ57R plasmid or PUCm-T easy vector).
4. Preparation of competent cells: Competent cells for the transfer of recombinant DNA will be prepared from pure cultures of *E.coli* (DH5α). This will be done using different methods like, CsCl method, electroporation, gene gun approach etc.

5. Identification of transformed cells: This will be done using blue-white selection.
6. Sequencing of the cloned PCR product: It will be carried out by dideoxynucleotide chain termination method using specific primers. The DNA sequence will be analyzed using the BLAST (Basic Local Alignment Search Tool) program of NCBI (National Centre for Biotechnology Information, USA) or PGDs (Plant Genome Database, pgd.org).

b) Designing SCAR primers – The specific SCAR primers will be designed from the sequence of cloned specific DNA fragments by using softwares such as primer 3 tool (http://biotools.unmassmed.edu), Oligo 6.0 (Molecular Biology insights, Inc. Cascade, CO, USA) on the basis of primer size Tm and G +C content.

3g) The specificity of SCAR primers will be verified by screening the different sex types among different populations for sex linked markers.

4. Expected outcome: Following outcome is expected after the completion of this work:

1. The molecular control of the sex expression in *V. wallichii* will be understood.

2. Sex specific molecular markers will be available for this species which would be helpful in the screening of plants even at early vegetative stage which is otherwise not possible.

3. It will be possible to adopt a scientifically more efficient method of selective collection and cultivation of specific sex type plants to obtain the active principle in better quantity and quality.

4. The information on diversity of sex distribution among populations will help in developing appropriate conservation measures for the species.

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